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A Preliminary Assessment of Silver Nanoparticle Inhibition of Monkeypox Virus Plaque Formation

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Abstract The use of nanotechnology and nanomaterials in medical research is growing. Silver-containing nanoparticles have previously demonstrated antimicrobial efficacy against bacteria and viral particles. This preliminary study utilized an in vitro approach to evaluate the ability of silver-based nanoparticles to inhibit infectivity of the biological select agent, monkeypox virus (MPV). Nanoparticles (10–80 nm, with or without polysaccharide coating), or silver nitrate (AgNO_3) at concentrations of 100, 50, 25, and 12.5 $\mu\text{g/mL}$ were evaluated for efficacy using a plaque reduction assay. Both Ag-PS-25 (polysaccharide-coated, 25 nm) and Ag-NP-55 (non-coated, 55 nm) exhibited a significant ($P \leq 0.05$) dose-dependent effect of test compound concentration on the mean number of plaque-forming units (PFU). All concentrations of silver nitrate (except 100 $\mu\text{g/mL}$) and Ag-PS-10 promoted significant ($P \leq 0.05$) decreases in the number of observed PFU compared to untreated controls. Some nanoparticle treatments led to increased MPV PFU ranging from 1.04- to 1.8-fold above controls. No cytotoxicity (Vero cell monolayer sloughing) was caused by any test compound, except 100 $\mu\text{g/mL}$ AgNO_3 . These results demonstrate that silver-based nanoparticles of approximately 10 nm inhibit MPV infection in vitro, supporting their potential use as an anti-viral therapeutic.

Keywords Nanoparticle · Monkeypox virus · Silver · Anti-viral therapeutic · Plaque reduction assay

Introduction

Nanotechnology is a growing field of science that utilizes the physico-chemical properties of nanomaterials as a means to control their size, surface area, and shape for use in multiple aspects of research and application in every day life. Nanomaterials are functionally defined as having a single-dimensional feature within the range of 1–100 nm and have been used to create various nanomaterials with unique properties superior to their bulk materials. In biological science and medicine, proposed uses for nanomaterials include drug delivery, biosensors, imaging, antimicrobials, and cancer therapeutics [1–6]. Nanomaterials have also been used in consumer products [7], as well as electronics, sensors, munitions, and propulsion technologies [8].

Monkeypox virus (MPV), an orthopoxvirus similar to variola virus, is the causative agent of monkeypox in many species of non-human primates and is endemic to central and western Africa [9]. Monkeypox virus is also a significant public health concern as it is a human pathogen with a clinical presentation similar to that of smallpox [9]. Like variola, MPV is considered a potential biological warfare threat and the development of various types of drugs and therapeutics against these viruses is ongoing [10].

The interaction of nanoparticles with microorganisms is a developing area of research that has included the evaluation of the antimicrobial capacity of certain silver-containing nanoparticles against vegetative bacteria and the human immunodeficiency virus (HIV)-1 [5, 6]. Previous research on the nanoparticle-HIV-1 interaction demonstrated that silver-containing nanoparticles inhibited HIV-1

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infectivity in vitro by binding to the disulfide bond regions of the CD4 binding domain within the gp120 glycoprotein subunit. Binding of these nanoparticles to the gp120 subunit appeared to be size dependent as particles greater than 10 nm were not observed attached to the viral envelope [6]. Based on this previous study, the use of silver-containing nanoparticles as an anti-viral therapeutic may be a new area of developing nanotechnology-based anti-viral therapeutics. Therefore, the purpose of this preliminary study was to utilize an in vitro approach to evaluate the ability of various silver-based nanoparticles to inhibit MPV infectivity.

Materials and Methods

Virus

The monkeypox virus Zaire strain (MPOX-Z, CDC isolate V79-I-005), originally obtained in 1979 from a fatally infected human in Zaire and shown to be fatal in cynomolgus monkeys [11], was used for testing. A working stock of MPV was prepared in serum-free Earle's Minimum Essential Medium (EMEM; ATCC, Manassas, VA) at a target concentration of 1×10^3 plaque-forming units (PFU)/mL.

Nanoparticles

Plasma gas-synthesized silver nanoparticles, 25 (Ag-NP-25), 55 (Ag-NP-55), and 80 (Ag-NP-80) nm, were a gift from Dr. Karl Martin (Novacentrix, Austin, TX). The Ag-NP were processed with hydrocarbons that prevent sintering, leaving a non-uniform hydrocarbon surface layer. The polysaccharide-coated silver nanoparticles, 10 (Ag-PS-10), 25 (Ag-PS-25), and 80 (Ag-PS-80) nm nanoparticles, were a gift from Dr. Dan Goia (Clarkson University, Center for Advanced Materials Processing, Potsdam, NY). The Ag-PS nanoparticles have a continuous polysaccharide (acacia gum) surface coating. All the silver nanoparticles were diluted in sterile, deionized water to 1 mg/mL. Silver nitrate (AgNO_3 ; Sigma, St. Louis, MO) was used as an antimicrobial control.

Efficacy Testing

All work was conducted under biosafety level 3 conditions. The efficacy of silver nanoparticles and AgNO_3 was determined using a plaque reduction assay similar to previously described methods [12]. Briefly, African green monkey kidney (Vero) cells were seeded in 12-well plates at a density of 3×10^5 cells/well in EMEM containing 10% fetal bovine serum (FBS; VWR International, West

Chester, PA) and cultured at 37 °C under 5% CO_2 for 24 h until the cells reached >90% confluency.

Each of the silver nanoparticles and AgNO_3 were serially diluted two-fold in serum free EMEM yielding concentrations of 200, 100, 50, and 25 $\mu\text{g/mL}$. Equal volumes (200 μL) of the working virus stock and each nanoparticle (or AgNO_3) concentration were combined, yielding nanoparticle and AgNO_3 concentrations of 100, 50, 25, and 12.5 $\mu\text{g/mL}$. This suspension also yielded a target virus concentration of 50 PFU/well. The resultant suspensions ($n = 3\text{--}6$ /test compound) were incubated at room temperature (20 ± 2 °C) for 15 min and 100 μL of each suspension was plated onto Vero cell monolayers in triplicate to each well of a 12-well plate. The plates were incubated at 37 °C under 5% CO_2 for 1 h with gentle rocking every 15 min. Approximately 1 mL of 0.7% methylcellulose (Sigma) in EMEM containing 10% FBS and supplements (non-essential amino acids, L-glutamine, penicillin/streptomycin; VWR International) was added to each well and the plates were incubated at 37 °C under 5% CO_2 for 72–76 h. Following incubation, cultures were stained with 0.065% crystal violet in phosphate-buffered saline (PBS) containing 6% formaldehyde (Sigma) for 15 min and then rinsed with PBS to remove excess crystal violet. The visualized plaques were manually counted and the total number of PFU/well determined. Parallel samples containing virus only (0 $\mu\text{g/mL}$ test compound) were used as a positive control to determine average PFU/well. Negative controls (blanks) consisted of serum-free EMEM without virus, but in the presence of the test compound.

Statistical Analysis

Data were expressed as mean number of PFU per well \pm standard deviation (SD). The one-way ANOVA and the *t*-test (MS Excel; Microsoft Corporation, Redmond, WA) were used for the data analysis. The one-way ANOVA was used to determine the effect of test compound concentration on the mean number of PFU/well. The two-sample *t*-test was used to compare the average number of PFU/well between the untreated positive controls (virus only; 0 $\mu\text{g/mL}$ test compound) and each treatment group. $P \leq 0.05$ was used as the level for significance.

Results

For all the concentrations tested, none of the nanoparticles induced any observed cytotoxicity that would have interfered with the plaque counting. However, the 100 $\mu\text{g/mL}$ AgNO_3 did cause cytotoxicity of the Vero cells, as these monolayers sloughed off the wells of the tissue culture

Table 1 Mean (\pm SD) PFU/well of monkeypox virus treated with silver nanoparticles or silver nitrate

	Treatment	Concentration ($\mu\text{g/mL}$)				
		100	50	25	12.5	0
	Ag-PS-10	10 \pm 3.2*	16 \pm 7.5*	16 \pm 4.7*	19 \pm 6.7*	– ^a
^a Not applicable	Ag-PS-25 ^c	58 \pm 7.4	50 \pm 3.8	47 \pm 5.7	42 \pm 5.4	–
^b Not determined due to observed cytotoxicity	Ag-NP-25	74 \pm 11	86 \pm 7.8	87 \pm 7.9	71 \pm 3.1	–
^c Significant effect of all test compound concentrations on mean PFU/well (one-way ANOVA; $P \leq 0.05$)	Ag-NP-55 ^c	72 \pm 5.7	67 \pm 3.1	51 \pm 7.4	41 \pm 3.8	–
	Ag-PS-80	71 \pm 13	75 \pm 6.7	67 \pm 7.0	61 \pm 7.0	–
	Ag-NP-80	50 \pm 16	47 \pm 15	40 \pm 16	36 \pm 15	–
	AgNO ₃	ND ^b	34 \pm 10*	33 \pm 5.2*	29 \pm 8.8*	–
[*] Mean value is significantly lower than control (t -test; $P \leq 0.05$)	Control	–	–	–	–	48 \pm 9.9
	Blank	0	0	0	0	0

plates, which precluded the ability to collect data for this concentration of AgNO₃.

The results of the efficacy testing for all nanoparticles including the positive control (AgNO₃) are provided in Table 1. One-way ANOVA analysis revealed that both Ag-PS-25 and Ag-NP-55 exhibited an overall significant ($P \leq 0.05$) dose-dependent effect of test compound concentration on the mean number of PFU/well. The Ag-PS-10 also exhibited a dose-response effect of nanoparticle concentration; however, this dose-dependent effect was not significant (one-way ANOVA; $P=0.055$) due to the fact that all Ag-PS-10 concentrations tested significantly reduced MPV plaque formation.

For all concentrations tested, both the Ag-PS-10 and AgNO₃ promoted a significant ($P \leq 0.05$) decrease in the number of observed PFU/well when compared to the untreated controls (Table 1). These decreases in MPV plaque formation ranged from 60 to 79% and 29 to 40%, respectively. Interestingly, the nanoparticles Ag-PS-25, Ag-NP-55, and Ag-NP-80 exhibited decreased MPV plaque formation at the lowest concentration tested (12.5 $\mu\text{g/mL}$); however, these decreases were not statistically significant. For some of the nanoparticle treatments, there was an observed increase in the mean number of MPV PFU/well, which ranged from 1.04- to 1.8-fold above controls (Table 1). Figure 1 shows representative increases and decreases in MPV plaques associated with various treatments.

Discussion

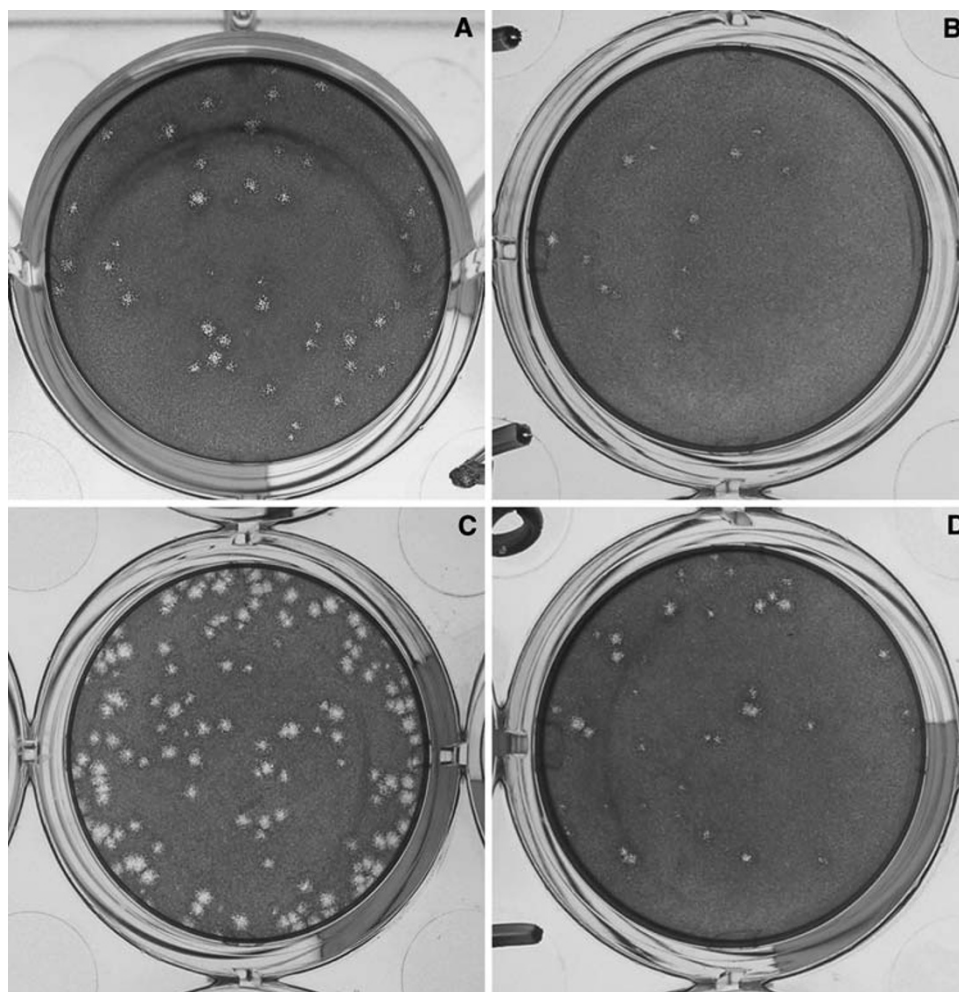
The present study shows that Ag-PS-10 and AgNO₃ were effective at reducing MPV-induced plaque formation in vitro at concentrations ranging from 12.5 to 100 $\mu\text{g/mL}$. These results are similar to a previous study in which nanoparticles with silver concentrations greater than 25 $\mu\text{g/mL}$ reduced HIV-1 infectivity below detection [6]. Moreover, this

reduction in HIV-1 infectivity was also size dependent, as only silver-containing nanoparticles ranging from 1 to 10 nm in diameter established a strong enough physical interaction with the gp120 glycoprotein of HIV-1 virions to inhibit viral binding to a host-cell [6]. With respect to all the nanoparticles tested in the present study, including the known antimicrobial AgNO₃, our results indicate that the silver-containing nanoparticles with a diameter of approximately 10 nm (Ag-PS-10) were the most effective at inhibiting MPV infectivity as demonstrated by the statistically significant reduction in MPV plaque formation at all concentrations tested. Although these data indicate that Ag-PS-10 and AgNO₃ decrease MPV plaque formation, the mechanism by which this inhibition occurs is not known and could involve blockade of host cell binding, disruption of host cell biochemical pathways, or both.

Poxvirus entry into a host cell can occur by endocytosis or direct fusion with the plasma membrane, which is followed by a regulated sequence of events leading to viral replication [13]. Currently, the specific cellular receptors or proteins involved in poxvirus fusion with the plasma membrane and subsequent cellular entry are not known. In the present study, the inhibition of MPV plaque formation could be due to the physical obstruction of virus–host cell binding by nanoparticles as previously observed with HIV-1 [6]. However, the internalization of metal-based nanoparticles by cultured cells and the subsequent changes in cellular biochemistry [14] suggest there is also a possibility for disruption of intracellular pathways that could ultimately attenuate viral replication. Understanding the mechanism(s) by which silver-containing nanoparticles exhibit anti-viral properties with respect to nanoparticle size, concentration, and cellular interaction would be valuable for future development of, or modification to, existing nanoparticle technologies to produce more effective nanoscale-based anti-viral therapeutics.

An interesting observation from the data in the present study was that the Ag-PS-25, Ag-NP-25, Ag-NP-55, and

Fig. 1 Representative monkeypox virus plaques in Vero cell monolayers in an untreated control (a), or following treatment with 50 $\mu\text{g/mL}$ of (b) Ag-PS-10, (c) Ag-NP-25, or (d) AgNO_3



Ag-PS-80 nanoparticles promoted an increase in the mean number of MPV PFU/well when compared to controls that was consistent for the 50 and 100 $\mu\text{g/mL}$ concentrations. A potential explanation for this may be due to nanoparticle agglomeration where the size of these agglomerates is variable and can change based upon the medium in which the nanoparticles are suspended [15]. Previous work has shown that silver (15 nm) and manganese (40 nm) nanoparticles tend to agglomerate and that these agglomerations can be found both intra- and extracellularly [14]. Therefore, it is possible that the nanoparticle agglomeration may potentiate or facilitate virus particle interaction or internalization within host cells, leading to an increase in the number of observed PFU. This is supported by that fact that none of the nanoparticles alone promoted cellular cytotoxicity, but defined plaques were observed when the nanoparticles (e.g., Ag-NP-25) were added to the cultured cells in the presence of virus (Fig. 1c). However, these results are preliminary and more research is needed to understand the silver nanoparticle–virus interaction and

associated mechanisms by which these nanoparticles affect virus infectivity and plaque formation.

The use of nanotechnology and nanomaterials in medical research is a fast-growing field in which the functional mechanisms of metal-based nanoparticles within a complex biological system are just beginning to be understood. Silver-containing nanoparticles have been shown to exhibit antimicrobial efficacy against bacteria and viral particles, supporting their use as potential therapeutics against biological agents. The present study demonstrates the feasibility of implementing the use, and characterizing the efficacy, of silver-based nanoparticles against MPV infection *in vitro*. However, for nanoparticles to be used in therapeutic or prophylactic treatment regimens, it is critical to understand the *in vivo* toxicity and potential for long-term sequelae associated with exposure to these compounds. Further research will be needed to answer these important questions, especially to design nanomaterials that exhibit a high degree of biocompatibility with a nominal negative effect on host cells.

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